EXHIBIT A

Docket No.: 4600-0109PUS1
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Susumu YAMAGUCHI et al.

Application No.: 10/513,593 | Confirmation No.: 7853

Filed: September 30, 2005 | Art Unit: 1794

For: BODY TASTE IMPROVER COMPRISING LONG-CHAIN HIGHLY UNSATURATED FATTY ACID AND/OR ESTER THEREOF AND VEGETABLE FAT COMPOSITION CONTAINING THE SAME | Examiner: C. A. Paden

DECLARATION SUBMITTED UNDER 37 C.F.R. § 1.132

Honorable Commissioner
Of Patents and Trademarks
P.O. Box 1450
Alexandria, VA 22313-1450

May 12, 2010

Sir:

I, Susumu Yamaguchi of the Oils and Fats Fundamental Technology Laboratory, J-Oil Mills Inc., Japan, do hereby declare the following:

I have attached a copy of my curriculum vitae to this Declaration.

I am manager of R&D department and have worked in this field for 18 years)

I am familiar with the above referenced patent application and the area of science dealing with body taste and the use of long-chain highly unsaturated fatty acids in food production. I have read and understand the subject matter of the Office Action of January 6, 2010.

The following comments are offered in support of the patentability of the instant invention.

1. The Examiner states that the claims are obvious over van Dorp (US 3,686,003) alone or in combination with Massie (US 6,344,225). Specifically the Examiner states that van Dorp discloses adding arachidonic acid to foods to produce a savory flavor. The Examiner recognizes that van Dorp does not use fat as a matrix for the flavoring, but that since fat is considered to be hydrophobic it would be expected to act as a solvent for arachidonic acid and could replace the hexane used by van Dorp. The Examiner also notes that Massie teaches the use of fat, particularly beef tallow as a carrier for flavor in frying oil. Based on these beliefs, the Examiner concludes that using arachidonic acid for producing a body-taste would be obvious. I strongly disagree.

2. To begin, it is well known in the art that slightly autooxidized arachidonic acid has a flavor resembling cooked chicken meat (see for example, page 356, second paragraph of the Introduction in Harkes and Begemann (1974) J. Am. Oil Chem. Soc. No. 51,pages 356-359; attached). It is also well known in the art that the flavor of chicken or other species-specific tastes, is generally lipid-derived and that these lipids may be degraded via thermal and oxidative reactions to have sensory effects (see, for example, page 1197, right column, lines 4-8 and 13-16 of Taylor and Larick (1995) J. Food Sci. 60(6):1197-1200; attached).

3. The examples in the van Dorp reference relating to altering the flavor of foods, such as the soup in Example 20, has chicken meat and chicken fat already present before the addition of arachidonic acid. In these cases, the soup is described as "having only a weak

flavor of chicken" (van Dorp, column 7, lines 45-49). The purpose of adding arachidonic acid is to give a "more marked chicken flavor" to the soup (van Dorp, column 7, lines 45-49). Van Dorp also discusses producing a similar soup of "excellent chicken flavor" where the chicken fat (3.0 g) is first mixed with 0.18 cc of 10% ethanol solution of arachidonic acid (90% pure) (van Dorp, column 7, liens 68-72).

4. It is also well known in the art that the composition of representative fatty acids is very different between chicken fat and vegetable oils, such as corn oil and canola oil. For example, while arachidonic acid is contained in chicken oil in an amount of about 0.5-1.5%, it is not substantially contained in vegetable oil.

5. The attached Table presents the volatile compounds that are generated via thermal and oxidative reactions and which are present in vegetable oil, as represented by corn oil, and arachidonic acid (shadowed columns). Clearly, the two oils have very different composition, sharing only 4 of a total of 41 substances. As can be seen from the "total flavor description" of corn oil versus arachidonic oil, to one skilled in the art this essentially means that vegetable fat and oil never produces a chicken flavor.

6. The present invention mixes an n-6 or n-3 long-chain highly unsaturated fatty acid with vegetable fat and oil in such a small amount (i.e. 10-10,000 ppm (1%)) followed by oxidization treatment.

7. Considering this in view of the third column in the attached Table, it is clear that the volatile compounds derived from arachidonic acid do not have a significantly lower odor

threshold and so their flavor is not especially stronger than that of the volatile compounds from vegetable oil. As a consequence, the addition of n-6 or n-3 long-chain highly unsaturated fatty acids such as arachidonic acid will not produce any chicken flavor in the vegetable fat and oil. Instead, a new total or mixed flavor is generated in the vegetable fat and oil that is different from the original flavor that is due to the volatile compounds derived from the arachidonic acid or other n-6 or n-3 long-chain highly unsaturated fatty acid which has been introduced.

8. As can be seen from the above, the vegetable fat and oil is never just a carrier for the n-6 or n-3 long-chain highly unsaturated fatty acid an/or an ester. The addition of this n-6 or n-3 long-chain highly unsaturated fatty acid and/or an ester will not simply emphasize a flavor that is already present in the vegetable fat and oil. Neither will the vegetable fat and oil simply take on the flavor of the added n-6 or n-3 long-chain highly unsaturated fatty acid an/or an ester.

9. The new flavor that is generated as the total or mixed flavor is called KOKUMI, which is a Japanese term that can be translated as "body-taste." The English term Kokumi has come to mean taste reinforcement accompanied by thickness, continuity and mouthfulness (see page 810, left column, lines 3-7 of Yamamoto et al. (2009) Chem. Senses 34:809-818; see also page 64, right column, lines 24-32 of FoodTechnology (August 2004) 58(8):56-69, both of which are attached). This definition is substantially the same as that presented in the present application on page 10-11 "Way of sensory test of potato shoe strings."

10. With respect to Massie, in my opinion the skilled artisan would have no reason to combine the Massie and van Dorp references to obtain the instant invention. Massie never discloses or suggests using n-6 or n-3 long-chain highly unsaturated fatty acid and/or ester to improve the body taste of the vegetable fat and oil.

11. To conclude, in my opinion the skilled artisan would not find the current application obvious in view of the disclosure in the van Dorp reference, with or without the disclosure in the Massie reference.

EXHIBIT A

Appl. No: 10/513,593

The undersigned hereby declares that all statements made herein based upon knowledge are true, and that all statements made based upon information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

DATED: 12. May. 2010 Susumu Yamaguchi

Susumu Yamaguchi

EXHIBIT A

Volatile compound[c,d]	Reported odor descripton[a,c]	Reported odor threshold in oil (mg/kg)[a] (mg/L)[b]	Reported aroma intensity[f*] scale from 1(low) to 3(high)	corn[d] AA[e] total flavor description	
				Nutty[e] Buttry Corny Burnt Rancid Painty Off flavor	Cooked chicken[e]
Ethane					
Propane					
Propanal					
Pentene		340[a]			
Pentane					
Propanal					
Pentene					
Hexane					
2-butenal		0.025[a]			
1-penten-3-ol					
Pentanal					
Heptane					
Pentenal	Painty,herbal	0.07[a]			
Pentanol					
Octen					
Hexanal	Fatty, green, fruity, cut grass, herbal, rancid, painty, crushed weeds	0.12[a] 0.3[b]	3		
Octen					
Octen					
t-2-hexanal					
Heptanal	weed, green, sour, sweaty, herbal, painty, rancid	0.055[a]			
c-2-heptanal					
t-2-heptenal					
1-octen-3-ol	mushroom-like	0.01[b]	2		
pentyl fran					
t,c-2,4-heptadienal	fatty, natty				
Octanal	Lime, grassy, citrus, sharp, heavy, candle-like, crushed weeds	1.5[a]			
t,t-2,4-heptadienal	fatty, nutty				
Octenal					
Nonanal	Green, soapy, rubbery,				
Dodecane					
t-2-decenal					
Decenol					
t,c-2,4-decadienal	soapy	0.004[b]	2-3		
t,t-2,4-decadienal	fatty	0.18[b]	2		
Undecenal					
1-octen-3-one	mushroom-like		3		
c-2-octenal	soapy		2		
t-2-octenal	soapy		2		
c-2-nonenal	soapy		2		
t-2-nonenal	soapy		2		
t,c-2,4-nonadienal	soapy		2		
t,t-2,4-nonadienal	fatty		2-3		
2,5-undecadienal	soapy		3		
t-4,5-epoxy-t-2-decenal	metaric, green	0.0013[b]	3		
t,c,c-2,4,7-tridecatrienal	egg-white-like, marine	0.18[b]	3		
t,t,c-2,4,7-tridecatrienal	animal, beefy		2-3		
t,t,t-2,4,7-	animal, pig-like		2		
unknown	green, metallic				
unknown	soapy, geranium-like				

a) JAOCS, 73, 1154-1160 (1996)
b) Lipids, 36, 749-756 (2001)
c) J. Agric. Food Chem. 49, 2959-2965 (2001)
d) JAOCS, 62, 1675- 1679 (1985)
e) JAOCS, 51, 358-359 (1974)
f) Frontiers of flavor science pp3-9 (2000)
g) JAOCS, 73, 157-166 (1996)
* Intensity perceived at the sniffing port, on a scale from 1(low) to 3(high)

EXHIBIT A

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J. Am. Oil Chem. Soc. 57. 356-359 (1974) EXHIBIT A

Identification of Some Previously Unknown Aldehydes in Cooked Chicken

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ABSTRACT

Aldehydes present in a flavor concentrate, obtained from cooked chicken were separated and isolated by means of gas liquid chromatography. Subsequently, they were converted into their 2,4-dinitrophenylhydrazones and identified by thin layer chromatography on Kieselguhr G-Carbowax 750 and Silica Gel G-$AgNO_3$ and by analysis after partial hydrogenation. Finally, they were compared with model substances. Besides the aldehydes which had been found earlier in cooked chicken the following new aldehydes were identified: 3-c-nonenal; 4-c-decenal; 2-t,4-c,7-c-decatrienal; 2-t,5-c-undecadienal; 2-t-dodecenal; 2-t,4-c-dodecadienal; 2-t,6-c- and 2-t,6-t-dodecadienal; 2-t-tridecenal; 2-t,4-c-tridecadienal; 2-t,4-c,7-c-tridecatrienal; and 2-t,4-c-tetradecadienal. Three of them, 4-c-decenal; 2-t,6-c-dodecadienal; and 2-t,4-c,7-c-tridecatrienal are typical breakdown products of arachidonic acid, and to a major extent also 2-t,5-c-undecadienal.

INTRODUCTION

During the last two decades many articles on chicken flavor have been published, and the compounds described have been summarized by Wilson and Katz (1). As to aldehydes, octadecanal was the longest chain found in the saturated group, 2-t-undecenal in the alkenals and 2-t,4-t-decadienal in the alkadienals. Many of the aldehydes present also have been found in beef fats (2,3), milk fat (4), and peanut oil (5).

About the origin of the chicken flavor and its possible precursors, e.g. the following statements can be made. Chicken flavor is produced during cooking, whereas leaf fat contributes very little to the chicken flavor (6,7). Chicken flavor precursors are extracted readily from raw meat by cold water (8). Slightly autoxidized arachidonic acid has a flavor resembling cooked chicken meat (9). The percentage of arachidonic acid in the phospholipid fraction is highest in the low fat tissues (white and dark meat) and lowest in the high fat tissues (depot fat) (10). Removal of the carbonyls from a chicken flavor concentrate results in a loss of chickeny flavor and intensification of the meaty or beef-like odor (11).

These statements suggest that the breakdown products of arachidonic acid play an important role in the development of the cooked chicken flavor. If this is so, only aldehydes with a long chain length and polyunsaturated should be considered, as many of the unsaturated aldehydes already found also can be derived from oleic and linoleic acid which are the main unsaturated fatty acids of the chicken lipid fraction.

EXPERIMENTAL PROCEDURES

Preparation of Chicken Flavor Concentrate

Starting from five freshly slaughtered chickens, the flavor concentrate was obtained as shown in Figure 1. The volatiles, obtained after degassing and collected in cooled U-tubes (12), were taken up in purified pentane and combined.

Isolation of Carbonyls

A chicken flavor concentrate is a very complex mixture which was confirmed by Nonaka, et al., (13) who counted ca. 227 peaks on the gas chromatogram. Moreover, he noted that many peaks could not be identified due to incomplete separation of the peaks and a continuous bleeding of high boiling material from the chromatographic column, by which the mass spectra could not be interpreted. Therefore, the flavor concentrate was separated into 27 fractions by gas liquid chromatography (GLC), as indicated in Figure 2. On the column 40 μliter concentrate was injected, and each fraction was trapped in a small U-tube which was cooled in a mixture of solid carbon dioxide and ethanol. This operation was repeated five times and each fraction always was trapped in the same U-tube. By rinsing with carbonyl free light petroleum, the contents of each tube were brought onto a small 2,4-dinotrophenylhydrazone (DNPH) reaction column (14). The DNPHs thus formed were eluted with light petroleum and further analyzed.

Identification of Carbonyls

As the total amount of the DNPH of each fraction was very small and as we wanted to get as much information as possible, the following sequence of analyses was used.

Partition thin layer chromatography (TLC) on Kieselguhr G-Carbowax 750: The DNPH of each tube was brought as a band on a Kieselguhr G chromatoplate impregnated with 33% Carbowax 750 analogous to the method used by Badings (15). Eluant was light petroleum. Using this system, DNPHs of saturated aldehydes with a chain length from C_1 to C_{16} can be separated. Therefore, these DNPHs were used as model substances to mark the place of the migration of the unknown DNPHs. There also is a separation into classes as the migration rate of the

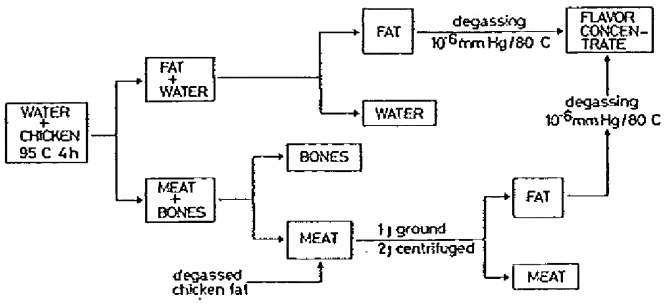

FIG. 1. Preparation of a chicken flavor concentrate.

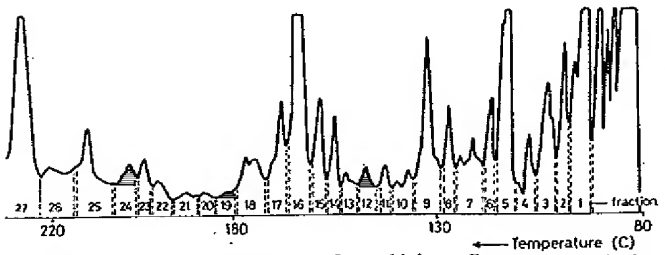

FIG. 2. Gas chromatogram of a chicken flavor concentrate. Column length: 2 m; internal diameter: 4 mm; stationary phase: silicon oil 20%; temperature: 1 C/min; nitrogen flow: 15 ml/min; support: Chromosorb W 60-100 mesh.

EXHIBIT A

TABLE I

Identification of Aldehydes Present in Chicken Flavor Fractions Isolated by Gas Liquid Chromatography (GLC)

GLC fraction no.	Carbowax 750 Band no.	Carbowax 750 R_f	γmax nm	$AgNO_3$ (R_f acetone = 1)	Carbowax R_f after partial hydrogenation[a]						Conclusion aldehyde		Identical with aldehyde	
1	1	C_6	358	1.6								C_6		C6
4	1	C_7	358	1.6								C_7		C_7
7	1	$C_{5.5}$	375	1.5		C_7					2	C_7	2t	C_7
8	1	$C_{3.5}$	387	1.5	$C_{5.5}$	C_7					2,4	C_7	2t,4c	C_7
9	1	$C_{6.5}$	374	1.5			C_8				2	C_8	2t	C_8
10	1	$C_{7.5}$	358	0.95				C_9			p[b]	C_9[c]	3c	C_9
	2	C_9	358	1.6								C_9		C_9
11	1	$C_{7.5}$	374	1.5				C_9			2	C_9	2t	C_9
12	1	$C_{8.5}$	358	0.7					C_{10}		p[b]	C_{10}[c]	4c	C_{10}
	2	C_{10}	358	1.6								C_{10}		C_{10}
13	1	$C_{5.5}$	387	1.5		$C_{7.5}$		C_9			2,4	C_9	2t,4c	C_9
14	1	$C_{8.5}$	374	1.5					C_{10}		2	C_{10}	2t	C_{10}
16	1	$C_{6.5}$	391	1.5			$C_{8.5}$		C_{10}		2,4	C_{10}	2t,4c	C_{10}
17	1	$C_{4.5}$	390	1.0	$C_{6.5}$		$C_{8.5}$		C_{10}		2,4,p[b]	C_{10}[c]	2t,4c,7c	C_{10}
	2	$C_{6.5}$	390	1.5			$C_{8.5}$		C_{10}		2,4	C_{10}	2t,4c	C_{10}
	3	$C_{7.5}$	374	1.0				$C_{9.5}$		C_{11}	2,p[b]	C_{11}[c]	2t,5c	C_{11}
	4	$C_{9.5}$	375	1.5						C_{11}	2	C_{11}	2t	C_{11}
19	1	C_8	375	0.8					C_{10}	C_{12}	2,p[b]	C_{12}[c]	2t,6c	C_{12}
	2	$C_{8.5}$	374	1.2					C_{10}	C_{12}	2,p[b]	C_{12}[c]	2t,6t	C_{12}
	3	C_{10}	374	1.5						C_{12}	2	C_{12}[c]	2t	C_{12}
20	1	C_{13}	358	1.6								C_{13}		C_{13}
21	1	$C_{8.5}$	390	1.5					C_{10}	C_{12}	2,4	C_{12}[c]	2t,4c	C_{12}
22	1	$C_{11.5}$	374	1.5						C_{13}	2	C_{13}[c]	2t	C_{13}
23	1	C_{14}	358	1.6								C_{14}		C_{14}
24	1	C_7	387	1.0				C_9	$C_{11.5}$	C_{13}	2,4,p[b]	C_{13}[c]	2t,4c,7c	C_{13}
	2	C_9	388	1.5					$C_{11.5}$	C_{13}	2,4	C_{13}[c]	2t,4c	C_{13}
25	1	C_{15}	358	1.5								C_{15}		C_{15}
26	1	$C_{10.5}$	388	1.5					$C_{12.5}$	C_{14}	2,4	C_{14}[c]	2t,4c	C_{14}
	2	C_{16}	359	1.5								C_{16}		C_{16}

[a]The R_f of the original 2,4-dinitrophenylhydrazone has been omitted.

[b]An isolated double bond is present but the position is unknown.

[c]Isolated for the first time.

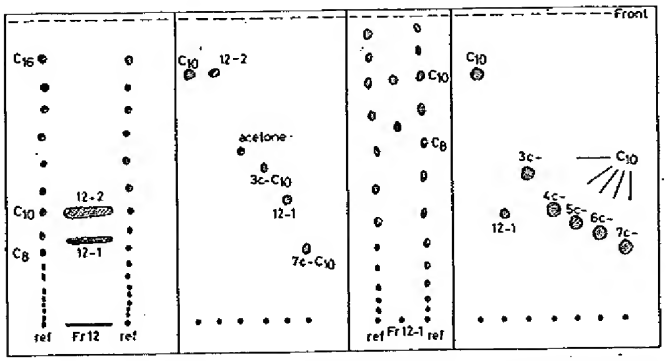

FIG. 3. Thin layer chromatograms of the 2,4-dinitrophenylhydrazones (DNPHs) of fraction 12. I, adsorbent: 33.3% Carbowax 750 on Kieselguhr G; mobile phase: light petroleum. Ref: DNPHs of C_1-C_{16} alkanals; II, adsorbent: 25% $AgNO_3$ on Silica Gel G; mobile phase: benzene; III, after partial hydrogenation; conditions as in I. Ref: DNPHs of C_1-C_{12} alkanals; and IV, conditions as in II.

DNPH decreases with increasing number of unsaturated double bonds in the aldehyde chain (16).

UV measurement: Each separated band was scraped off and eluted with chloroform. The wavelength of maximum absorption was measured to determine whether a saturated or a conjugated unsaturated aldehyde with one or two double bonds was present.

Argentation TLC on Silica Gel G-$AgNO_3$: A small amount of each band was further analyzed on a Silica Gel G plate impregnated with 25% $AgNO_3$ and using benzene as eluant (17). With this system, DNPHs containing one or more isolated double bonds can be separated from the saturated and conjugated unsaturated ones. The relative migration of the DNPHs was measured with respect to that of acetone-DNPH, as values of ca. one or less are an indication that the DNPH possesses a *cis*-isolated double bond, whereas values between 1 and 1.5 point to presence of a *trans*-isolated double bond.

Partial hydrogenation of the DNPH: If there are indications of an unsaturated DNPH, a small amount was hydrogenated partially with palladium on calcium carbonate. This technique (18), offers two data, viz the chain length of the straight chain aldehyde and the number of double bonds. The chain length follows from the R_f of that spot, which, after hydrogenation, is the most mobile and which corresponds with that of one of the reference DNPHs of the saturated aldehydes on a Kieselguhr G-Carbowax 750 plate.

By partial hydrogenation, a number of DNPHs is obtained, the unsaturation of which in the aliphatic chain decreases. The number of spots minus the original spot corresponds with the number of double bonds present in the original aliphatic chain of the DNPH.

RESULTS AND DISCUSSION

The amount of DNPHs was too small to carry out a double bond location analysis by oxidative degradation (19). Therefore, we could only locate the position of the isolated double bond by comparing the unknown DNPH with a model substance, but, on account of the various data already obtained, the choice of the model substance was much easier.

In the last column of Table I, the aldehydes are given which are equal to the isolated ones with respect to their retention time on GLC and their DNPH behavior on TLC. In fractions 2, 3, 5, 6, 15, 18, 27 no DNPHs could be detected. The aldehydes (Table I) discussed below were isolated from cooked chicken for the first time.

3-c-Nonenal (fraction [Fr] 10, band [b] 1): As linoleic acid is present in the neutral lipids and phospholipids and arachidonic acid in the phospholipids, the presence of 3-c-nonenal is understandable.

4-c-Decenal (Fr 12, b1): In the first instance, it was not obvious to predict the position of the isolated *cis*-double bond as, according to the generally accepted route proposed by Farmer, et al. (20), this aldehyde could not be derived from the polyunsaturated fatty acids normally occurring. In Figure 3, the identification procedure is shown. However, later on, Badings (21) detected 4-c-decenal as one of the autoxidation products of arachidonic acid. So it is most likely that 4-c-decenal, as found in cooked chicken, has been derived from arachidonic acid.

2-t,4-c,7-c-Decatrienal (Fr 17, b1): This aldehyde can be derived from linolenic acid which occurs in small amounts in the depot fat. It also has been identified in other ω3,6,9 unsaturated fatty acids by Meyboom and Stroink (22).

2-t,5-c-Undecadienal (Fr 17, b3): This aldehyde can be a breakdown product of linoleic acid, as well as of arachidonic acid. As an α-methylene group is known to be more reactive when it is flanked on either side with a double bond (arachidonic acid) than on one side (linoleic acid), it is more obvious that this aldehyde comes from arachidonic acid.

2-t-6-c-Dodecadienal (Fr 19, b1): One of the autoxidation products of arachidonic acid is 3-c,6-c-dodecadienal. Aldehydes with a 3-*cis*-double bond isomerize easily to the 2-*trans*-configuration, as shown by Grosch and Schwartz (23) who isolated 2-t,6-c-nonadienal from cucumber homogenates incubated with ($U^{14}C$)-linolenic and ($U^{14}C$)-linoleic acid. They did not find 3-c,6-c-nonadienal which would be formed from linolenic acid but 2-t,6-c-nonadienal instead.

2-t,6-t-Dodecadienal (Fr 19, b2): We have no explanation for its formation.

2-t,4-c,7-c-Tridecatrienal (Fr 24, b1): This aldehyde is most likely a breakdown product of arachidonic acid.

The other aldehydes isolated for the first time from cooked chicken are 2-t-dodecenal (Fr 19, b3); 2-t,4-c-dodecadienal (Fr 21, b1), already tentatively identified (24); 2-t-tridecenal (Fr 22, b1); 2-t,4-c-tridecadienal (Fr 24, b2); and 2-t,4-c-tetradecadienal (Fr 26, b1). All these aldehydes cannot be predicted on theoretical grounds to be derived from the common polyunsaturated fatty acids present in chicken meat and fat.

Although, according to the GLC separation on silicone oil, we might expect two other breakdown products typical of arachidonic acid viz 2-t,5-c,8-c-tetradecatrienal in fraction 24 or 25 and 2-t,6-c,9-c-pentadecatrienal in fraction 26 or 27, we could not detect them in these fractions.

Some examples about the effect of these aldehydes upon chicken flavor are given in two patents (9,25).

ACKNOWLEDGMENTS

M.W. Langelaan, P.W. Meyboom, J.B.A. Stroink, and J.P. Ward synthesized the model substances.

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EXHIBIT A

Volatile Content and Sensory Attributes of Supercritical Carbon Dioxide Extracts of Cooked Chicken Fat

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ABSTRACT

Volatile and sensory profiles were generated for extracts of cooked chicken fat obtained with supercritical carbon dioxide at 10.3 MPa, 20.7 MPa, and 31.0 MPa at 40°C. Volatiles (318 total) were quantitated and 77 were identified. Concentrations of total volatiles, 7 compound classes, and 63 individual compounds were affected by treatments, generally increasing with decreasing extraction pressure. All extracts had higher concentrations than unextracted control. Total volatiles were concentrated 12-fold. Six sensory notes differed by treatment. Chicken fat aroma and flavor intensities were greater for all extracts than control and increased as pressure decreased. Total volatiles, 5 classes, and 42 individual compounds correlated with chicken fat aroma and/or flavor.

Key Words: chicken fat, sensory, volatiles, supercritical CO_2

INTRODUCTION

THE DEMAND FOR LOW-FAT PRODUCTS which often have inherent flavor disadvantages, especially in meats area require development of flavor concentrates for potential use in such products. Supercritical fluid extraction has potential for producing natural flavor concentrates while using excess lipid by-products of the poultry industry.

Supercritical fluids are heated and pressurized beyond the fluid-specific critical temperature and pressure (Bott, 1982; Calame and Steiner, 1982). They have solvating ability like liquids and diffusivity and mass transfers similar to gases (Brogle, 1982; Caragay, 1981; de Filippi, 1982; Hardardottir and Kinsella, 1988). Carbon dioxide is a popular supercritical fluid because it is low cost, nonflammable, nontoxic, and has relatively low critical temperature (31.1°C) and pressure (7.38 MPa). Commercial utilization of this technology includes decaffeination of coffee beans, and obtaining hops extracts and spice and fruit juice essential oils. Total extractions, removal of specific components, fractionation of fats and oils, and concentration of aroma and flavor compounds have been reported (Rizvi et al., 1986).

de Haan and de Graauw (1990) concentrated flavor compounds from milk fat up to 50-fold. Vapor pressure differences between volatiles and nonvolatile triglycerides facilitated extraction. Concentration of raw beef fat volatiles was demonstrated by Merkle and Larick (1994) with noted increases in volatile concentrations as extraction pressure decreased. Higher molecular weight volatiles became more soluble in the supercritical fluid as extraction pressure increased (Shimoda et al., 1994).

Over 300 volatile compounds have been identified from chicken systems, but chicken flavor is associated primarily with hydrocarbons, aldehydes, enals, ketones, and sulfur compounds. Aldehydes, enals, and sulfur-containing compounds have been most important. Minor et al. (1965) associated carbonyls with "chickeny" flavor and sulfur compounds with "meaty" flavor. Specifically, 2-alkenals including hexenal, heptenal, octenal, nonenal, undecenal, and dodecenal as well as aldehydes like octanal, nonanal, and decanal along with decadienal and γ-dodecalactone have been indicated to have potential impact on chicken-specific aroma and flavor (Gasser and Grosch, 1990; Pippen and Nonaka, 1960; Ramarathnam et al., 1991).

Species-specific notes are generally lipid-derived while meaty notes are lean-derived (Dwivedi, 1975; Hornstein and Crowe, 1960; 1963; Minor et al., 1965; Shahidi et al., 1986). Differences between species' fatty acid profiles and resulting carbonyls may be responsible for the lipid influence (Allen and Foegeding, 1981; Gray et al., 1981; Kim Ha and Lindsay, 1990; Ramarathnam et al., 1991; Ramaswamy and Richards, 1982; Rubin and Shahidi, 1988; Shahidi et al., 1986; Wong et al., 1975a,b). Dietary lipids are a primary determinant of poultry fatty acid deposition (Jen et al., 1971; Schuler and Essary, 1971). Lipids may dissolve or adsorb and later release important aroma/flavor compounds or be degraded via thermal and oxidative reactions to have sensory effects (Dwivedi, 1975; Moody, 1983; Selke et al., 1975; Shahidi et al., 1986).

Our objectives were to study supercritical fluid extraction to concentrate volatile compounds of chicken fat and to characterize important compounds for chicken sensory notes.

MATERIALS & METHODS

Sample preparation

Raw chicken fat was collected from random processing lots at the halving machine from a commercial processor, manually freed of nonlipid material and ground once through a 0.95 cm plate. Portions (500g) were weighed and vacuum-packaged in low permeability bags (20 × 28 cm P641B clear pouches; Cryovac Corp., Duncan, SC) and overwrapped with polyethylene coated freezer paper. Samples were stored at −20°C and thawed at 2–4°C prior to use. Prior to extraction, fat was heated in a convection oven (30 min) to 80°C in 500 mL Pyrex glass beakers to melt the triglyceride portion and create a substrate mimetic of roasted chicken fat. An unextracted portion was placed in a Pyrex tube (16 mm × 125 mm), flushed with N_2 gas, sealed with a teflon-lined cap, frozen at −10°C and retained as a control.

Extraction

A Superpressure model 46-13421-2 supercritical fluid extractor (Newport Scientific, Jessup, MD) equipped with a 69.0 MPa double end diaphragm compressor was used to fractionate the cooked chicken fat. Aliquots (250g) were immobilized between 2 plugs of glass wool and loaded into a 0.845L (internal volume) stainless steel extraction vessel. Vessel temperature was maintained at 40°C via an internal thermocouple and temperature controller with heat tape wrapped around the outside of the vessel. Stainless steel transfer lines were wrapped with insulation to maintain temperature within the system. Continuous extractions were carried out at 10.3, 20.7, and 31.0 MPa, using SC-CO_2 at 10–15 L/min to a total flow volume of 500 L ambient CO_2 measured using a flow totalizer. Extracts were collected in a 500 mL Pyrex round bottom flask upon fluid depressurization at ambient temperature. Extracts were transferred to Pyrex tubes (16 mm × 125 mm), flushed with N_2, sealed with a teflon-lined cap, and frozen at −10°C until analysis.

Volatile quantitation

Extracts and unextracted control were melted in a 60°C water bath. Samples (300 mg) were placed into another tube and 1026 ng internal standard, 2,3,4-trimethylpentane, was added. The tube was sealed and vortexed. Aliquots (100 mg) were placed between 2 plugs of pesticide grade glass wool in a 9 mm × 85 mm glass tube. With a 6-port sample

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EXHIBIT A

Table 1—Volatile class concentrations of supercritical carbon dioxide extracts of chicken fat

	Concentration (ppm)			
Pressure (MPa)	0.0	10.3	20.7	31.0
Class				
Enals	1.37^{c}	15.75^{a}	10.65^{ab}	4.06^{bc}
Aldehydes	1.43	16.32	11.96	6.09
Ketones*	0.11^{b}	3.21^{a}	2.82^{a}	1.06^{b}
Lactones*	0.16^{d}	4.43^{a}	1.77^{b}	0.85^{c}
Alkanes	0.14	1.70	1.06	0.69
Branched alkanes	4.26^{b}	26.79^{a}	14.45^{b}	9.10^{b}
Alcohols	0.05^{b}	3.60^{a}	2.04^{ab}	0.80^{b}
Acids	0.52	3.47	2.18	0.04
Alkenes	0.53	4.40	3.16	2.27
Halogenates	0.06^{b}	0.70^{a}	0.54^{a}	0.40^{ab}
Aromatics	0.06^{c}	1.65^{a}	1.15^{ab}	0.57^{bc}
Heterocyclics	0.00	0.04	0.00	0.07
Total*	9.94^{c}	123.80^{a}	76.63^{ab}	37.49^{bc}

^{abcd} Means in same row with same letter or with no superscript do not differ significantly ($P \leq 0.05$).
* ($P \leq 0.01$).

valve in the no-flow position, the tube was positioned in an External Closed Inlet Device (Scientific Instrument Service, River Ridge, LA). Volatiles were stripped from the sample via this method of direct thermal desorption followed by cold trapping for 5 min. Temperatures were: inlet 150°C, valve 160°C, and carrier lines 170°C. Volatiles were flushed onto a nonpolar 30m DB-5 fused silica capillary column (J & W Scientific, Folsom, CA) with internal diameter 0.32 mm and film thickness 1.0 micron. The column was in a Varian 3700 gas chromatograph (Varian, Palo Alto, CA) equipped with a flame ionization detector and maintained with head pressure of 1.13 psi, helium carrier gas flow rate of 5.73 mL/min, and split ratio of 20.12:1. An oven temperature program of −30°C to 290°C at 4°C/min was used with a 1 min hold at −30°C. Data were analyzed using the Maxima 820 Chromatography Workstation (Millipore, Waters Chromatography Division, Milford, MA). Quantitation of volatiles was based on relative peak areas compared to peak area of internal standard.

Volatile identification

For identification via electrical ionization, 10.3 MPa and 20.7 MPa extract samples (200 mg or 550 mg plus 1 mL deionized water) were placed into a sampling tube of a Tekmar LSC-3 headspace concentrator. Samples were purged at 110°C for 14 to 20 min followed by 5 min desorption onto a 30m DB-5 fused silica capillary column with internal diameter 0.32 mm and film thickness 1.0 micron within a Hewlett Packard 5985 gas chromatograph/mass spectrometer. Oven temperature program was identical to that used in volatile quantitation and ionization potential was 70 eV. Scan range was 40–300 atomic mass units. For chemical ionization with methane gas, samples (500 mg) were purged 14 min, desorbed 5 min, and scanned over a 40–300 atomic mass unit range with the same equipment set-up and an ionization potential of 230 eV.

For more efficient concentration of higher molecular weight compounds, a modified liquid/liquid extraction was used (Likens and Nickerson, 1964). Cooked fat (100g) and deionized water (150 mL) were placed into a 500 mL Pyrex round bottom flask with boiling chips and hooked to the long arm of a water-jacketed codistillation apparatus. The short arm was fitted to a 100 mL Pyrex round bottom flask containing boiling chips and 75 mL Optima grade methylene chloride (Fisher Scientific, Fair Lawn, NJ). Heating mantles beneath each flask were used to heat samples to boiling. Once both sides had begun condensing at the top of the still, codistillation was allowed for 6 hr. After codistillation and cooling, remaining solvent phase containing entrapped volatiles was drained from the distillation apparatus and added to the 100 mL Pyrex round bottom flask. A Buchi rotary evaporator was used to concentrate solvent fraction to ≈ 1 mL. Flask was washed 3 times with 1 mL methylene chloride and the washings were added to original 1 mL of concentrate. Combined sample was concentrated under a gentle stream of N_2 to 0.1 mL prior to GC-MS analysis.

Samples (1-2 µL) from liquid/liquid extractions were injected directly onto a 30m DB-5 capillary column in a Hewlett Packard 5985 gas chromatograph/mass spectrometer under conditions noted and scanned over a 40–500 atomic mass unit range. Similar conditions were used for chemical ionization with methane gas with an ionization potential of 230 eV as for previous chemical ionization analyses. Volatile identifications were based on instrument retention times for previously identified compounds and GC-MS with both electrical and chemical ionization methods and comparison to NIH/EPA (1978) reference spectra.

Sensory analysis

Separate extractions for each treatment under identical conditions, using food-grade carbon dioxide, were carried out to collect samples for sensory analysis. Extracts were flushed with nitrogen gas and stored at −10°C.

Melted extracts and control were presented as a 30% solution in mineral oil to an established 7 member trained flavor profile panel (ASTM, 1981; Caul, 1957). Prior chicken training sessions (3) included samples of melted (as described above) chicken fat, 10.3 MPa extract, pure mineral oil, and cooked chicken meat. Treatment samples were evaluated individually using a 14 point intensity scale (1 = not detectable, 14 = strong) on 5 aroma and 9 flavor notes previously selected by panelists during training or dictated by experimental goals. Consensus scores were developed through discussion by all panelists following independent evaluation. Panel evaluation was completed in 2 sessions with 4 samples/session.

Experimental design and statistical analysis

Extractions and volatile profiles were replicated 3 times on different samples of fat for the 4 treatments, 3 extracts and unextracted control. Extractions and sensory profiles were replicated twice on fat from a single source for the 4 treatments. Volatile concentrations and sensory responses were analyzed via analysis of variance using a randomized complete block design with replicates as blocks. Waller-Duncan k-ratio t-tests were used to separate means (SAS Institute, Inc., 1990). Volatile concentrations and sensory responses were averaged across replicates and correlated using Pearson correlation coefficients (SAS Institute, Inc., 1990). All reported significant differences and correlations are at $P \leq 0.05$, unless otherwise noted.

RESULTS & DISCUSSION

Volatile quantitation and identification

Quantitated volatile compounds numbered 318 of which 99 were grouped into 12 compound classes—enals, aldehydes, ketones, lactones, alkanes, branched alkanes, alcohols, acids, alkenes, halogenated compounds, aromatic hydrocarbons, and heterocyclics. Of the 99 compounds, 77 were identified. All enals, aldehydes, alkanes, acids, and aromatic hydrocarbons and some ketones, lactones, alcohols, alkenes, halogenated compounds, and similar heterocyclics have been noted in other chicken samples (Gasser and Grosch, 1990; Katz et al., 1966; Shahidi et al., 1986). Thermal breakdown of fatty acids and oxidation of unsaturated lipids could yield compounds representative of all volatile classes (Ellis et al., 1961; Hoffman, 1962; MacLeod and Seyyedain-Ardebili, 1981) except aromatic hydrocarbons and halogenated alkanes. They likely came from the breakdown of aromatic amino acids in the original adipose tissue or feed constituents and entrapped water, respectively. The largest numbers of classified volatiles were branched alkanes (38), enals (12), and aldehydes (9) followed by alkenes (8), alcohols (6), ketones (6), aromatic hydrocarbons (5), halogenated compounds (4), lactones (4), alkanes (3), acids (3), and heterocyclics (1). An additional 11 volatile compounds (2-propenal; pentane; carbon disulfide; 2-methylpentane; nitromethane; 3-methylpentane; 2-methyl-1-pentene; butanal; hexane; 1,2-dichloroethene; and chloroform) were identified in extracts. However they were not quantitatable due to the coelution of internal standard carrier solvent, hexane.

By concentration, branched alkanes, aldehydes, and enals were predominant (Table 1) in all treatments. Treatments affected total volatile concentration which was higher for all extracts vs control and, among extracts, increased with decreasing extraction pressure. Total volatiles were concentrated over 12-fold in 10.3 MPa extract vs control. Similarly, concentrations of enals, ketones, lactones, branched alkanes, alcohols, and halogenated and aromatic compounds were affected by treatment. Concentration of aldehydes followed a similar trend but varia-

Table 2—Pearson correlation coefficients for identified volatiles and flavor notes that differed by treatment

Peak No.	Identification	Chicken fat flavor	Astringent flavor
50	4-Methyloctane	0.972;0.028[a]	**[b]
66	2,2,4-Trimethylheptane	**	0.979;0.020
69	2,2,6-Trimethylheptane	**	0.982;0.018
70	2-Heptanone	0.975;0.025	**
88	1-Nonen-3-ol	**	**
89	2,3-Octanedione	**	**
91	2-Pentylfuran	**	0.998;0.002
108	(2,5 or 2,6)-Dimethylundecane	**	**
130	Nonanal	**	0.999;0.001
137	(2,2,6 or 2,2,7)-Trimethyldecane	**	**
147	2(E)-Nonenal	**	0.999;0.001
155	3,3-Dimethylundecane	**	0.972;0.028
164	Decanal	**	0.984;0.016
165	2,4-Nonadienal	**	0.992;0.008
201	2-Undecenal	**	0.967;0.033
211	2,5-Dimethyldodecane	**	0.998;0.002
244	2,6-Di-*t*-butyl-4-methylphenol	**	**
264	Tetradecanal	**	0.959;0.042
273	γ-Dodecalactone	**	0.951;0.049
274	2-Pentadecanone	**	0.987;0.013
275	5-Propyltridecane	**	0.977;0.023
277	δ-Dodecalactone	**	**
290	Hexadecanal	**	0.970;0.031
300	2-Heptadecanone	**	**
306	δ-Tetradecalactone	**	**

[a] Correlation coefficient; level of significance.
[b] Not significant (P≤0.05).

Table 3—Aroma and flavor responses of supercritical carbon dioxide extracts of chicken fat

	Sensory response[d]			
	Pressure (MPa)			
Note	0.0	10.3	20.7	31.0
Oxidized aroma	1.1[b]	3.5[a]	3.4[a]	3.3[a]
Chicken fat aroma	1.8[b]	3.8[a]	3.1[a]	2.8[ab]
Mineral oil aroma	1.6	1.5	1.7	1.4
Cooked aroma	1.1	1.2	1.1	1.1
Meaty aroma	1.0	1.0	1.0	1.0
Oxidized flavor*	1.0[b]	4.3[a]	4.1[a]	4.5[a]
Chicken fat flavor	2.3[c]	4.6[a]	4.1[ab]	3.8[b]
Mineral oil flavor	1.9	1.7	1.6	1.8
Metallic flavor	1.0	1.6	1.3	1.3
Cooked flavor	1.3	1.8	1.5	1.1
Sour flavor	1.6	1.8	1.7	1.8
Astringent flavor	1.8[b]	2.4[a]	2.1[ab]	1.9[b]
Meaty flavor	1.1	1.1	1.0	1.0
Oxidized aftertaste*	1.0[b]	3.8[a]	3.6[a]	4.0[a]

[abc] Means in same row with same letter or with no superscript do not differ significantly (P≤0.05).
[d] Scored on a 1–14 scale.
* (P≤0.01).

Table 4—Identified volatiles correlated with chicken fat aroma and flavor

Chicken fat aroma	Chicken fat flavor
Pentanal	1,1,1-Trichloroethane
Hexanal	2,2,4-Trimethylhexane
2-Octene	2-Octene
4-Methyloctane	2-Methyl-2-heptene
3,6-Dimethylheptane	4-Methyloctane
(1,3 or 1,4)-Xylene	2,6-Dimethylheptane
2,2,5-Trimethylheptane	3,5-Dimethylheptane
2,2,4-Trimethylheptane	(1,3 or 1,4)-Xylene
2,2,6-Trimethylheptane	2-Heptanone
2-Heptanone	
Xylene isomer	
Heptanal	
2(E)-Decenal	
Tetradecane	
2-Methyl-6-propyldodecane	

tion between replicates, partially due to variation in fat source, limited significance (P≤0.10). Classes were concentrated from 6-fold for branched alkanes to 66-fold for alcohols with enals and aldehydes concentrated 11-fold. The concentrations achieved were related to both the nature of the class of compounds with regard to volatility and polarity as well as specific compounds identified. The concentration of aldehydes, enals, and lactones was expected as they should be quite soluble in SC-CO_2. The branched alkanes are more volatile and soluble than their straight chain counterparts. More polar alcohols should be less soluble but several of those identified had unsaturation which would increase solubility. Halogenated compounds noted consisted of very short carbon chains. The aromatic compounds were all monocyclic with the only phenol having considerable branching to enhance solubility (Dandge, 1985). Merkle and Larick (1994) documented similar results with supercritical fluid extracted raw beef fat.

Individual compounds affected by treatments numbered 63 of which 25 were identified—7 branched hydrocarbons, 4 aldehydes, 4 ketones, 3 enals, 3 lactones, 2 aromatics, 1 alkene, and 1 alcohol (Table 2). All compounds except 2 (2,3-octanedione and 2,6-di-*t*-butyl-4-methylphenol) were in greater concentration in all extracts vs control and concentration increased with decreasing extraction pressure. These 2 compounds were more concentrated in all extracts than in the control, with the greatest concentration in the 20.7 MPa extract. 2,6-di-*t*-Butyl-4-methylphenol was identified in fatty acid analyses and its concentration increased (P≤0.10) with decreasing extraction pressure (Taylor, 1994). Ramarathnam et al. (1993) identified this compound as unique to chicken compared to beef and pork.

Nonanal, 2(E)-nonenal, 2(E),4(E)-nonadienal, 2-undecenal, and γ-dodecalactone have been identified as potent odorants with mainly "fatty" and "tallowy" descriptions (Gasser and Grosch, 1988, 1990; Ullrich and Grosch, 1987). Nonanal, 2-undecenal, and γ-dodecalactone were indicated as having specific odor potency in chicken over bovine species. Decanal, also, had notably higher concentration in chicken than in beef and pork (Ramarathnam et al., 1991).

The largest concentrating effect of extraction was for 2-heptadecanone and trimethyldecane (2,2,6- or 2,2,7-). Individual enals and aldehydes, noted as important to chicken attributes, were concentrated 17-fold for nonanal to 68-fold for 2,4-nonadienal and γ-dodecalactone increased 29-fold.

Hexanal was in highest concentration in all treatments. It increased (P≤0.10) with decreasing extraction pressure though all extracts had more than did the control. 2(E),4(Z)-Decadienal was next highest in all treatments except the 10.3 MPa extract which had 2-undecenal at the second highest concentration. Hexanal is a likely product of oxidation of linoleic acid or further oxidation of preformed 2,4-decadienal via an epoxide intermediate. 2,4-Decadienal could arise from carbon-carbon bond cleavage on the chain of the 9-hydroperoxide arising from linolenic acid (18:3). Cleavage of the carbon-carbon bond on the acid side of the 8-hydroperoxide radical from the oxidation of oleic acid (18:1) could account for the presence of 2-undecenal (Fennema, 1985).

All pressure treatments had sufficient solvating capacity in the SC-CO_2 to result in volatile concentration over the unextracted control. The volatile concentration trend was due to the supercritical fluid's decreased solubilization at lower pressures for less volatile and higher molecular weight fatty acids and triglycerides. Thus, volatiles were selectively extracted and concentrated. At higher pressures, more types of compounds would compete for position in the supercritical fluid phase. Merkle and Larick (1994) similarly achieved concentration of beef fat volatiles at lower pressures. Near the critical point of CO_2, on the steeper portion of the solubility curve, is where the most effective separations should occur (Allada, 1984).

Sensory analysis

Five aroma and 9 flavor notes were evaluated by trained panelists (Table 3). All notes, except meaty descriptors, were placed on the ballot by panelists during training. The meaty note was

EXHIBIT A

added to ascertain if a purely lipid substrate would produce meaty sensations.

Among aroma notes, oxidized and chicken fat notes differed with treatment. All extracts elicited greater response intensity of these notes from panelists than did unextracted control. A trend of increasing intensity with decreasing pressure among extracts was noted, especially for the chicken fat aroma note. No meaty aroma and very little cooked aroma were detectable in any treatment, indicating the inability of a cooked lipid substrate to produce volatiles required for meaty notes, concurring with reported studies (Dwivedi, 1975; Hornstein and Crowe, 1960, 1963; Minor et al., 1965; Shahidi et al., 1986).

Among flavor notes, oxidized, chicken fat, astringent, and oxidized aftertaste were greater in all extracts than in controls with the exception of the 20.7 MPa and 31.0 MPa treatments for the astringent note. Chicken fat and astringent flavor increased among extracts with decreasing extraction pressure with a very clear trend for the chicken fat note. The meaty note was barely detectable in any sample, on average, and not by all panelists in any case. Clearly, intensity of aroma and flavor responses, especially for oxidized and chicken fat notes, increased with extractions. Their values increased several levels by extraction and usually in sequence, indicating the close association of such perceptions. Oxidized aroma and flavor, often undesirable attributes, increased but we could not determine from the data if the increase reached unacceptable levels as no hedonic testing was undertaken. The 30 % solution presented to panelists was well in excess of the usage level common for flavor concentrates but was required for adequate numerical data to facilitate distinction of differences.

Correlation of volatile concentrations and sensory notes

Qualitatively, trends for chicken fat aroma and flavor and oxidized aroma were very similar to concentration trends for total volatiles, branched alkanes, aldehydes, and enals. Total volatiles, aldehydes, alkanes, alkenes, halogenated compounds, and aromatic hydrocarbons correlated with chicken fat aroma. A negative correlation for heterocyclics and mineral oil aroma was noted. Alkenes and halogenated compounds correlated with chicken fat flavor. Lactones, alkanes, and branched alkanes correlated with metallic flavor. Acids correlated with cooked flavor. All classes except ketones and halogenated and heterocyclic compounds correlated with astringent flavor.

Correlations for concentrations of enals (r=0.935; 0.065), ketones (r=0.935; 0.065), branched alkanes (r=0.936; 0.064), and alcohols (r=0.945; 0.055) with chicken fat aroma approached significance at noted levels. Likewise, correlations of concentrations of aldehydes (r=0.925; 0.075), alkanes (r=0.936; 0.064), and aromatic hydrocarbons (r=0.928; 0.072) with chicken fat flavor approached significance. Lack of correlation between aldehydes, long considered indicators of oxidation, and oxidized aroma and flavor notes may be attributable to the large variation between replicates for aldehyde concentration, perhaps due to initial differences in fatty acid profiles for replicate fat sources.

Fifteen individual identified compounds consisting mainly of branched alkanes and aldehydes correlated with chicken fat aroma (Table 4) as did an additional 22 unknown compounds. Noted compounds of interest with correlations between concentrations and chicken fat aroma bordering on significance included 2-hexenal (r=0.926; 0.074), 2(E)-heptenal (r=0.920; 0.080), octanal (r=0.944; 0.056), nonanal (r=0.940; 0.060), 2(E)-nonenal (r=0.926; 0.074), and 2,4-nonadienal (r=0.913; 0.087). Chicken fat flavor correlated with 9 individual identified compounds (Table 4) of which 5 previously correlated with chicken fat aroma. These volatiles consisted mainly of branched alkanes and alkenes. An additional 8 unknown compounds correlated with chicken fat flavor, 7 of which previously correlated with chicken fat aroma. Pentanal (r=0.939; 0.061), hexanal (r=0.947; 0.053), and 2,6-di-*t*-butyl-4-methylphenol (r=0.920; 0.080) concentrations approached significant correlations with chicken fat flavor. A similar trend occurred for oxidized and chicken fat notes and correlation between compounds like pentanal and hexanal, which have correlated with oxidized notes (Evans et al., 1971), with chicken fat aroma and flavor. This indicated that oxidized sensations, within a range of acceptability, may be important in chicken-specific sensory attributes, and the descriptors may not be entirely separable by panelists.

Alkanes, ketones, and saturated alcohols from lipid oxidation are not very important to aroma and flavor (Forss, 1969). Aldehydes and related compounds are very important due to their higher concentrations and lower threshold values for perception and have been a primary focus for monitoring oxidation (Evans et al., 1971; Shahidi et al., 1986). All treatments yielded hexanal and decadienal concentrations in excess of some published flavor threshold values in similar media. However, most published thresholds were determined in single compound systems that did not consider possible synergies and masking of other components which may apply in a system with more than 300 quantitated volatiles.

In order to reduce the numerous correlations, specific attention was directed to correlations between volatile concentrations that differed by treatment (Table 2) and sensory attributes that differed by treatment. Of the 25 such identified compounds and 2 aroma notes the only correlations demonstrated were for concentrations of 4-methyloctane (r=0.971; 0.029); 2,2,4-trimethylheptane (r=0.987; 0.013); 2,2,6-trimethylheptane (r=0.985; 0.015); and 2-heptanone (r=0.977; 0.023) with chicken fat aroma. Lack of obvious correlations that were expected based on previous results for compounds like nonanal, 2(E)-nonenal, decanal, and 2(E)-undecenal was partially due to variation across replicates in fat source and extent of oxidation during cooking and extraction.

CONCLUSIONS

AROMA AND FLAVOR VOLATILES from cooked chicken fat, including classes of volatiles like aldehydes and enals that have been linked to species-specific notes were concentrated by supercritical fluid extraction with carbon dioxide. Concentration was most efficient at the lowest pressure, near the critical point. Sensory characteristics of extracts were affected by changes in volatile profiles. Similarities between identified volatiles correlated with chicken specific aroma and flavor, and published correlations for volatiles and oxidized notes, indicates that oxidized attributes were important in chicken flavor.

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—Continued on page 1204

EXHIBIT A

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Taste Preference and Nerve Response to 5′-Inosine Monophosphate Are Enhanced by Glutathione in Mice

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Abstract

Previous human sensory evaluation studies have shown that glutathione (GSH) enhances deliciousness, accompanied by thickness, mouthfulness, and continuity feeling, which is known as "kokumi" in Japanese, in an umami solution containing monosodium glutamate and 5′-inosine monophosphate (IMP). We conducted behavioral and electrophysiological experiments to explore possible interactions of taste effectiveness between GSH and umami substances in mice. The 2-bottle preference test revealed that the mice preferred GSH at concentrations ranging from 1 to 10 mM. When GSH was added to IMP or a mixture of IMP and monopotassium glutamate (MPG), the mice showed increased preference for these solutions over the individual IMP or the binary mixture of IMP and MPG in both short-term and long-term tests. The addition of GSH to MPG, however, did not increase preference. Neural responses of the chorda tympani and glossopharyngeal nerves to the mixture of IMP and GSH showed synergism, whereas synergism was not observed in the mixture of MPG and GSH in either taste nerve. Another behavioral study with the use of the conditioned taste aversion paradigm showed that aversions to MPG generalized moderately to GSH, but aversions to GSH did not generalize to MPG. The present study suggests that GSH enhances preference for umami solutions containing 5′-ribonucleotide rather than glutamate. On the basis of these results, we discuss possible receptors involved for the action of GSH.

Key words: electrophysiology, food additives, kokumi, preference, synergism, taste

Introduction

Deliciousness has an important role to play in enhancing consumption of food. Japanese people use the word "koku" on a daily basis when they evaluate the deliciousness of food. They often use this word in phrases such as, "this food is very delicious because of the koku in it." Koku is a conceptual word used for edibles implying strong deliciousness accompanying "thickness," "continuity," and "mouthfulness" in the flavors and textures (Ueda et al. 1990). Thickness refers to rich complexity, continuity refers to long-lasting sensory effects or an increase of aftertaste, and mouthfulness refers to sensory reinforcement or the increment of sensation throughout the whole mouth. Koku can be induced by rich chemical compositions contained in foodstuffs. It is said, for example, that cheddar cheese aged 9 months has more koku than that aged 2 months because the former has rich chemical reactions or decomposition products. Likewise, vintage wines have more koku than young wines such as Beaujolais Nouveau because the former have rich compositions, and soup cooked 6 h has more koku than soup cooked 1 h, for the same reason. Thus, koku is the most appropriately used when we enjoy the odor, texture, and color as well as the taste of food containing complex compounds after maturation.

The term "kokumi" ("mi" refers to taste in Japanese) has been suggested by previous scientists (Ueda et al. 1990, 1994, 1997; Fuke and Konosu 1991) when they refer to the concept

of koku in terms of taste component only rather than more complex components mentioned above, and this is the term we use in the present study. Although the definition of kokumi has not yet been accepted scientifically, it is noted here that kokumi does not refer to an independent taste quality like umami but instead refers to taste reinforcement accompanied again by thickness, continuity, and mouthfulness. In this case, however, these terms may be expressed in more specific ways. One way to describe thickness, continuity, and mouthfulness for human taste evaluation is as follows: 1) thickness refers to increased taste intensity evaluated 5 s after tasting, 2) continuity or long-lasting taste refers to persistent taste intensity measured 20 s after tasting, and 3) mouthfulness refers to the increment of taste sensation throughout the whole mouth (N. Miyamura, personal communication). Kokumi is used not only by Japanese but also, recently, has begun to be used by some European researchers (Dunkel et al. 2007; Toelstede et al. 2009) and by well-known American chefs (Kasabian D and Kasabian A 2005).

If you add a specific key substance instead of complex compounds to food as a seasoning and obtain a similar taste reinforcement effect, the substance can be called a kokumi-inducing substance. Among possible kokumi-inducing substances, such as glycogen, fat, oil, alliin, glutathione (GSH), sulfur-containing compounds, some specific peptides, amino acids, heated products of gelatin, and tropomyosin (Maga 1983; Ueda et al. 1990, 1994, 1997; Fuke and Konosu 1991; Kuroda and Harada 2004; Dunkel et al. 2007; Toelstede et al. 2009), GSH is a food candidate originally investigated by Ueda et al. (1997). GSH (L-γ-glutamyl-L-cysteinylglycine) is a tripeptide with glutamic acid, cysteine, and glycine that is widely included in foodstuffs such as meat, seafood, and wine. In a human sensory test, Ueda et al. (1997) reported through their simplified experimental paradigm that this peptide increased flavor characteristics of an umami solution containing 0.05% (about 1 mM) each of monosodium glutamate (MSG) and 5′-inosine monophosphate (IMP), but it did not affect the intensity of basic tastes, such as sweetness, saltiness, sourness, and umami. They reported that the increased flavor (or enhanced deliciousness) of the umami solution could be expressed by such terms as continuity, thickness, and mouthfulness, which are collectively called kokumi (or "kokumi flavor" or "kokumi taste," depending on the researchers), as described above.

The physiological mechanisms of kokumi are still a matter of speculation, for example, there are no answers to the question of whether kokumi is elicited among the chemical ingredients of food by a similar synergistic effect as that occurs in mixtures of umami substances (Yamaguchi 1967; Rifkin and Bartoshuk 1980; Kawamura and Kare 1987; Li et al. 2002). Yamaguchi (1987, 1998) also showed that umami was very important in increasing the deliciousness of food. To our knowledge, there is no report about the taste characteristics of GSH studied by the use of electrophysiological and behavioral techniques in animals. In the present study, therefore, we designed behavioral and electrophysiological experiments to examine possible interactions of taste effectiveness between GSH and umami substances in C57BL/6 mice. Part of the present study has been reported in abstract form (Watanabe and Yamamoto 2004).

Materials and methods

General procedure

Animals

Adult male C57BL/6-CrSLC mice, 8 weeks old at the beginning of the experiment, were used. They were housed in individual home cages in a temperature- (25 °C) and humidity (60%)-controlled room on a 12:12 h light/dark cycle. Animals had free access to food (dry pellets, MF) and tap water, except when deprived for training and testing as described below. All the experiments were carried out following the Guidelines for Ethical Treatment of Laboratory Animals in Osaka University and Asahi University.

Behavioral experiment

On the first day, the mice were put on a schedule of water deprivation for 8 h/day. The training period was from the second to the sixth days. In this period, each animal was trained to drink distilled water (dw) from 2 bottles. After the training period, the 2-bottle preference test was carried out. Each of the 2 bottles filled with a different taste stimulus (or dw) was presented simultaneously to each mouse on each test day. The order of presentation of test stimuli was randomized. The positions of the 2 bottles were switched every 24 h of the 48-h test session to avoid positional preference in the long-term test, and the positions of the bottles were switched every 1 min of the 10-min presentation period in the short-term test. The volume of intake for each solution in each bottle was measured.

Electrophysiological experiment

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg of body weight), and supplementary injections were given as needed to maintain a surgical level of anesthesia. A tracheal cannula was implanted, and the animal was secured by a head holder. The chorda tympani (CT) nerve was cut near its entrance into the tympanic bulla and dissected free from the underlying tissues. The glossopharyngeal (GL) nerve was also dissected free and cut near its entry to the posterior lacerated foramen. An indifferent electrode was positioned nearby in the wound. The whole-nerve activities were amplified, displayed on an oscilloscope, and monitored with an audio amplifier. The amplified signal was passed through an integrator with a time constant of 0.3 s and displayed on a slip chart recorder.

Taste nerve responses to test stimuli were recorded. Each stimulus solution and rinsing water flowed for 15 s at a constant flow rate (0.5 ml/s) controlled by a syringe pump at room temperature (25 ± 2 °C). The magnitude of the whole-nerve response was measured as the height of the integrated response from the baseline at 10 s after the onset of stimulation to avoid the tactile effects. Responses to taste stimuli were expressed as relative magnitudes of responses, when the magnitude of response to 0.1 M NH_4Cl was taken as the standard.

Experiment 1: preference test between GSH and dw

A total of 14 mice were used. Mice were subjected to the 2-bottle preference test between GSH and dw. Concentrations of GSH were 0.1, 0.3, 1, 3, 10, and 30 mM. We compared the total volume of intake per 30 g body weight for 48 h. The degree of preference was expressed as a preference score (=intake of GSH/sum of intake of GSH and dw).

Experiment 2: long-term preference for umami solutions with and without GSH

A total of 20 mice were used. They were divided into 2 groups and were subjected to a long-term 2-bottle preference test between dw and several taste solutions, including GSH, umami substances, and their mixtures. As taste stimuli, 1 mM GSH, 0.1 M monopotassium glutamate (MPG), 1 mM IMP, and their mixtures were used for one group and 1 mM GSH, 0.01 M MPG, 0.01 M IMP, and their mixtures were used for another group. It is noted here that the mixture contains the same concentrations of the individual components. We compared the total volume of intake per 30 g body weight for 48 h.

Experiment 3: short-term preference for umami solutions with and without GSH

A total of 10 mice were used. They were put on a schedule of water deprivation of 20 h/day. Each animal was placed in a test box and given free access to dw from 2 drinking bottles with stainless steel spouts for 10 min. Each spout contained a ball at the tip for the purpose of preventing spillage. We switched the bottles manually at the alarm sound of a timer set every 1 min. Supplemental water was available for 3 h in the home cage. After this training for a week, animals were subjected to a short-term 2-bottle preference test between dw and several taste solutions, including GSH, umami substances, and their mixtures. As taste stimuli, 1 mM GSH, 0.1 M MPG, 1 mM IMP, and their mixtures were used. We compared the volume of intake per 30 g body weight for 10 min.

Experiment 4: long-term preference test after denervation

A total of 24 mice were used. They were randomly divided into 4 groups ($n = 6$, each): naive control mice and mice with transection of either the bilateral chorda tympani nerve (CTx) or bilateral glossopharyngeal nerve (GLx) and mice with transection of both nerves (CTx + GLx). Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg). The ear ossicles through which the CT is running were crushed. The GL under the hypoglossal nerve was excised by tweezers. After the suture ligature, the mice were injected with penicillin G sodium (100 mg/kg) to avoid infection. All mice were allowed 6 days of postoperative recovery prior to any experimental manipulation. Each group was subjected to the long-term 2-bottle preference test between one of the taste solutions and dw. As taste stimuli, 0.1 M MPG with or without 1 mM GSH and 1 mM IMP with or without 1 mM GSH were used. The solutions were presented randomly to each mouse. The preference score was calculated for each taste solution in each animal, and the mean of the group was compared with each other. After the experiment, histological sections of the tongue were examined microscopically.

Experiment 5: conditioned taste aversion test

A total of 24 mice were used. They were randomly divided into 4 groups ($n = 6$, each) consisting of 2 experimental groups, GSH–LiCl and MPG–LiCl, and 2 control groups, GSH–NaCl and MPG–NaCl.

The mice were put on a schedule of water deprivation of 20 h/day. On the first training day, each animal was placed in a test box and given free access to dw for 1 h from a single drinking tube via a circular window. Supplemental water was available for 3 h in the home cage. The spout of polyethylene tubing (4 mm inner diameter) was located 2 mm outside the window. This arrangement prevented the spout from coming into contact with the animals' lips. Licks were detected by a lickometer equipped with a photo sensor. From the second to the fifth days, the training time was reduced from 1 h to 30 min. During this period, the animal was trained to drink dw on an interval schedule, consisting of 20-s presentations of dw with 30-s intertrial intervals, resulting in 30–50 trials during each 30-min session. On the sixth day, each animal was given access to either 0.1 M MPG for MPG–LiCl group or 0.01 M GSH for GSH–LiCl group as the conditioned stimulus and then given an intraperitoneal injection of 0.15 M LiCl (2% of the body weight) as an unconditioned stimulus that induces malaise with gastrointestinal distress. Control mice in MPG–NaCl group and GSH–NaCl group were injected with physiological saline instead of LiCl after ingestion of the MPG and GSH solutions, respectively. The seventh day was a recovery day. On the eighth day, the number of licks of each of the test stimuli was counted for 10 s after the first lick of each stimulus. Each test solution was presented randomly. The interval between each test solution was 30 s. The mean number of licks was obtained for each of the test stimuli in each mouse. Test stimuli were dw, 0.1 M MPG, 0.01 M GSH, 0.01 M IMP, 0.5 M sucrose(S), 0.1 M NaCl (N), 0.01 M HCl (H), and 0.0001 M quinine hydrochloride (Q).

Experiment 6: recording of CT and GL responses to taste stimuli

A total of 12 mice were used for recording of CT (6 mice) and GL (6 mice) responses to test stimuli. As test stimuli, 0.01 M GSH, 0.1 M MPG, 0.01 M IMP, 3 kinds of binary mixtures, such as 0.1 M MPG and 0.01 M IMP (MPG + IMP), 0.1 M MPG and 0.01 M GSH (MPG + GSH), 0.01 M IMP and 0.01 M GSH (IMP + GSH), and a trinary mixture of 0.1 M MPG, 0.01 M IMP, and 0.01 M GSH (MPG + IMP + GSH) were used. Note that the concentrations of GSH and IMP were 10 times higher than those used for the behavioral experiments because we wanted enough responses to these stimuli for quantitative analyses.

The synergistic effects were shown as the potentiation ratio (response to a mixture solution/arithmetic sum of responses to the individual stimuli in the mixture). A ratio exceeding 1.0 suggests the occurrence of synergism.

Statistical analyses

All data were analyzed using STATISTICA (Ver 5.5) software, and a result was considered significant if $P < 0.05$.

Results

Preference for GSH (Experiment 1)

Mean amounts of intake ± standard error (SE) (milliliter for 48 h per 30 g body weight) for 0.1, 0.3, 1, 3, 10, and 30 mM GSH versus dw were 7.8 ± 1.5 and 6.4 ± 0.7, 6.6 ± 0.5 and 5.4 ± 0.4, 10.1 ± 0.9 and 4.1 ± 0.5, 8.8 ± 0.9 and 3.6 ± 0.3, 10.0 ± 0.9 and 3.7 ± 0.4, and 6.8 ± 0.8 and 4.7 ± 0.5, respectively. The corresponding mean reference scores ± SE are shown in Figure 1. The preference scores at concentrations ranging from 1 to 10 mM were about 0.7, and these values were statistically significantly ($P < 0.001$, t-test) higher than the score 0.5 level. Preference scores for 0.1, 0.3, and 30 mM GSH stayed near the level of 0.5 ($P > 0.05$).

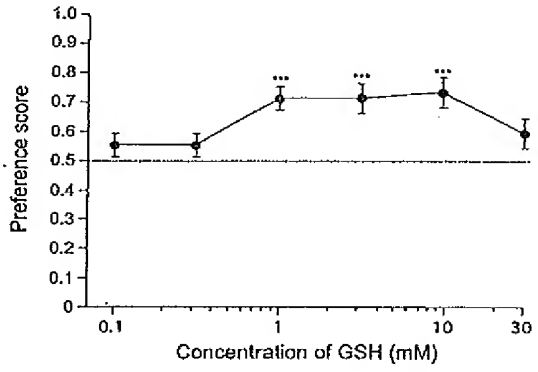

Figure 1 Mean preference scores ± SE for 6 concentrations of GSH. Asterisks indicate a significant difference; ***$P < 0.001$, t-test.

Long-term (48 h) preference test (Experiment 2)

Figure 2 shows the volume of intake (per 30 g body weight) for dw and 2 types of umami substances and their mixtures in the long-term 2-bottle preference test. When the volume of intake for 0.1 M MPG was compared with that of dw, MPG was preferred over dw significantly ($P < 0.05$, t-test) (Figure 2A). The binary mixture containing 0.1 M MPG and 1 mM GSH (MPG + GSH) was also preferred over dw ($P < 0.001$) (Figure 2B). However, when the volume of intake for MPG and that for MPG + GSH were compared, there was no significant difference between these 2 solutions ($P > 0.05$) (Figure 2C).

IMP solution (1 mM) was preferred over dw ($P < 0.05$) (Figure 2D). The binary mixture of 1 mM IMP and 1 mM GSH (IMP + GSH) was strongly preferred ($P < 0.001$) (Figure 2E). When the volume of intake for IMP and that for IMP + GSH were compared, the mixture was preferred over IMP ($P < 0.001$) (Figure 2F).

The binary mixture of 0.1 M MPG and 1 mM IMP (MPG + IMP) was preferred over dw ($P < 0.001$) (Figure 2G). When 1 mM GSH was added to this mixture, this new mixture (MPG + IMP + GSH) was greatly preferred over dw ($P < 0.001$) (Figure 2H). When the volume of intake

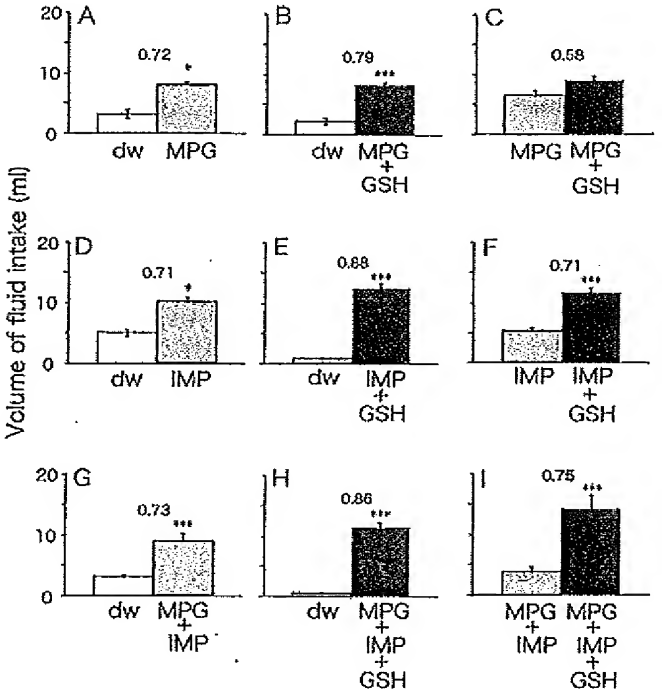

Figure 2 Mean volume of intake ± SE per 30 g body weight per 48 h for dw, 1 mM GSH, 0.1 M MPG, 1 mM IMP, and their mixtures in the long-term 2-bottle preference test. A pair of liquids in each graph was presented simultaneously for 48 h. Preference scores are shown in each graph. Asterisks indicate a significant difference; *$P < 0.05$, ***$P < 0.001$, t-test.

for MPG + IMP and that for MPG + IMP + GSH were compared, the latter was more preferred than the former ($P < 0.001$) (Figure 2I).

To examine the above finding that the addition of 1 mM GSH to 1 mM IMP, but not to 0.1 M MPG, increased preference, we did the same preference test with 1 mM GSH, 0.01 M IMP, and 0.01 M MPG. We obtained essentially the same result. As shown in Figure 3, when GSH was added to IMP, the mice showed increased preference to this mixture over IMP alone ($P < 0.01$, t-test). However, when the volume of intake for MPG and that for MPG + GSH were compared, there was no significant difference between these 2 solutions ($P > 0.05$).

Short-term (10 min) preference test (Experiment 3)

Figure 4 shows the volume of intake (per 30 g body weight for 10 min) for dw and 2 types of umami substances and their mixtures in the short-term 2-bottle preference test. When the intake of 0.1 M MPG was compared with that of dw, MPG was preferred over dw significantly ($P < 0.01$, t-test) (Figure 4A). However, when the volume of intake for MPG and that for MPG + GSH were compared, there was no significant difference between these 2 solutions ($P > 0.05$) (Figure 4B), which is consistent with the result obtained in the long-term test. IMP solution (1 mM) was preferred over dw ($P < 0.05$) (Figure 4C). When the volume of intake for IMP and that for IMP + GSH were compared, the mixture was preferred over IMP alone ($P < 0.001$) (Figure 4D), indicating similar results to those of the long-term test.

Long-term preference test after denervation (Experiment 4)

We examined the effect of the bilateral transection of either one or both of the CT and GL on the long-term preference. Because the CT and GL innervate taste buds on the anterior and posterior tongue, respectively, the transection of these nerves would reduce a substantial portion of gustatory information to the brain, although taste buds on the nasoincisal, palatal, and pharyngeal regions are spared. The transection was confirmed by verifying microscopically the loss of taste buds on the tongue.

Figure 5 shows mean preference scores for MPG with and without GSH (Figure 5A) and those for IMP with and without GSH (Figure 5B) in naive control mice and mice with transection of the CT (CTx), GL (GLx), and both CT and GL (CTx + GLx). Two-way (Nerve × GSH) analysis of variance (ANOVA) for MPG revealed significant main effect of Nerve, $F(3, 38) = 0.006$, $P < 0.01$, but no significant main effect of GSH and a Nerve × GSH interaction. On the other hand, the ANOVA for IMP revealed significant main effects of Nerve, $F(3, 38) = 7.63$, $P < 0.001$, and GSH, $F(1, 38) = 61.14$, $P < 0.001$, but no Nerve × GSH interaction. Further analysis of the data using Tukey's honestly significant difference (HSD) test showed that the preference scores for IMP with GSH were statistically significantly larger than those for IMP without GSH in control, CTx, and GLx mice, but there was no significant difference in CTx + GLx mice (Figure 5B).

Conditioned taste aversion test (Experiment 5)

Figure 6 shows generalization of aversion across 8 test stimuli including dw after establishment of aversions to either 0.1

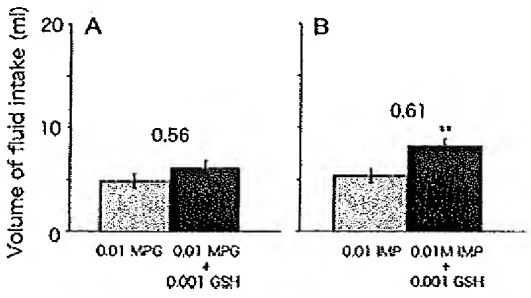

Figure 3 Mean volume of intake ± SE per 30 g body weight per 48 h for 0.01 M MPG and 0.01 M IMP with or without 1 mM GSH in the long-term 2-bottle preference test. A pair of liquids in each graph was presented simultaneously for 48 h. Preference scores are shown in each graph. Asterisk indicates a significant difference; $^{**}P < 0.01$, t-test.

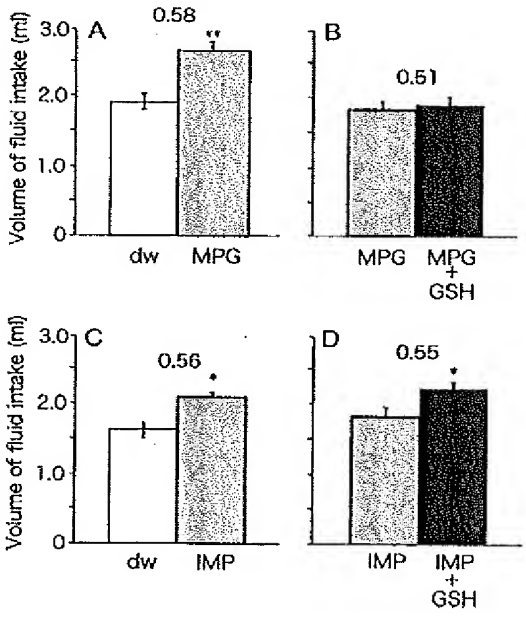

Figure 4 Mean volume of intake ± SE per 30 g body weight per 10 min for dw, 1 mM GSH, 0.1 M MPG, 1 mM IMP, and their mixtures in short-term 2-bottle preference test. A pair of liquids in each graph was presented simultaneously for 10 min with their positions changed every 1 min. Preference scores are shown in each graph. Asterisks indicate a significant difference; $^{*}P < 0.05$, $^{**}P < 0.01$, t-test.

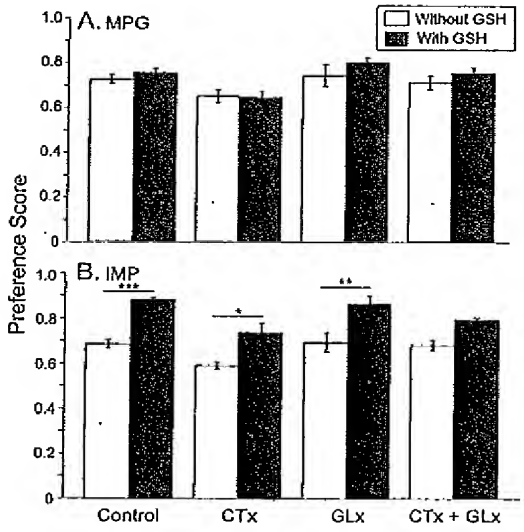

Figure 5 Mean preference scores ± SE for 0.1 M MPG with or without 1 mM GSH (A) and for 1 mM IMP with or without 1 mM GSH (B) in control and denervated mice. CTx, GLx, and CTx + GLx denote that only the CT, only the GL, and both nerves were transected, respectively, before the long-term preference test. Asterisks indicate a significant difference; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Tukey's HSD test.

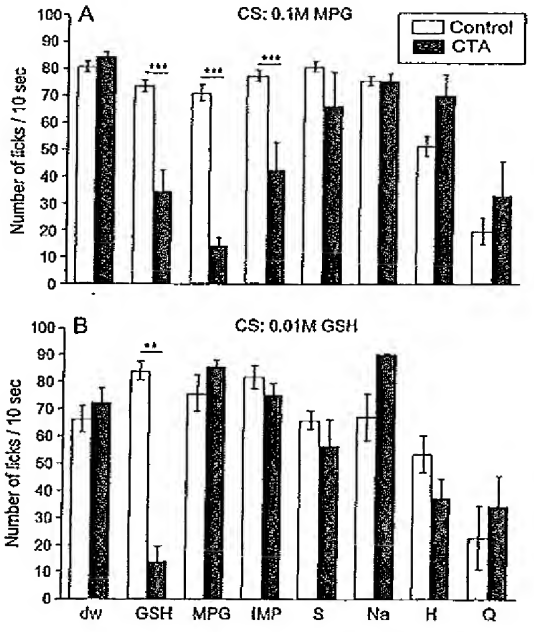

Figure 6 Mean numbers of licks ± SE for 8 test stimuli after conditioned taste aversions to 0.1 M MPG or 0.01 M GSH used as the conditioned stimulus (CS) in experimental group (CTA) and saline-injected control group (control). Suppression of licking was shown to MPG, GSH, and IMP after aversive conditioning to MPG, whereas suppression was shown only to GSH after aversive conditioning to GSH. $**P < 0.01$, $***P < 0.001$, Tukey's HSD test.

M MPG or 0.01 M GSH as expressed by mean numbers of licks during the first 10 s after the first lick. The mice conditioned to avoid MPG showed decreased licks to GSH and IMP as well as MPG. On the other hand, the animals conditioned to GSH showed decreased licks only to GSH, that is, no significant difference was observed to other taste stimuli and water between control and conditioned taste aversion (CTA) mice.

A 2-way (Group × Solution) ANOVA for conditioning to MPG revealed significant main effects of Group, $F(1, 112) = 28.07$, $P < 0.001$, and Solution, $F(7, 112) = 25.03$, $P < 0.001$, and a Group × Solution interaction, $F(7, 112) = 13.83$, $P < 0.001$. Post hoc analysis of the data using Tukey's HSD test showed that the numbers of licks to GSH, MPG, and IMP were significantly ($P < 0.001$) smaller than those in control group. A 2-way (Group × Solution) ANOVA for conditioning to GSH revealed significant main effects of Group, $F(1, 92) = 3.96$, $P < 0.05$, and Solution, $F(7, 92) = 15.13$, $P < 0.001$, and a Group × Solution interaction, $F(7, 92) = 13.03$, $P < 0.001$. Post hoc analysis of the data using Tukey's HSD test showed that the number of licks to GSH was significantly ($P < 0.01$) smaller than that in control group.

Taste nerve responses (Experiment 6)

Figure 7 shows sample records for umami, GSH, and their mixtures. Enhanced responses to the mixtures of GSH and umami substances as well as the enhanced response to the mixture of MPG and IMP were noted in both CT and GL. Quantitative analyses for these mixture effects are shown in Figure 8.

Figure 8A shows the relative responses to GSH, umami substances, and their mixtures in the CT. The mean magnitudes of responses (±SE, $n = 6$) to 0.1 M MPG, 0.01 M IMP, and 0.01 M GSH were 0.42 ± 0.11, 0.14 ± 0.01, and 0.36 ± 0.04, respectively. In the mixture solutions, the magnitudes of responses to MPG + IMP, MPG + GSH, IMP + GSH, and MPG + IMP + GSH were 0.98 ± 0.12, 0.60 ± 0.04, 1.10 ± 0.12, and 1.01 ± 0.15, respectively. Potentiation ratios for MPG + IMP, MPG + GSH, IMP + GSH, and MPG + IMP + GSH were 1.94 ± 0.26, 0.87 ± 0.16, 2.26 ± 0.13, and 1.19 ± 0.26, respectively. Responses to both MPG + IMP and IMP + GMP were significantly higher than those to the arithmetic sum of the individual components ($P < 0.05$ and $P < 0.01$, respectively, t-test), indicating the existence of synergism. There was no significant difference between the response to MPG + GSH and the arithmetic sum of the MPG and GSH responses ($P > 0.05$).

Figure 8B shows the relative responses to GSH, umami substances, and their mixtures in the GL. The mean magnitudes of responses (±SE, $n = 6$) to 0.1 M MPG, 0.01 M IMP, and 0.01 M GSH were 0.56 ± 0.05, 0.23 ± 0.02, and 0.47 ± 0.13, respectively. In the mixture solutions, the magnitudes of responses to MPG + IMP, MPG + GSH, IMP + GSH, and

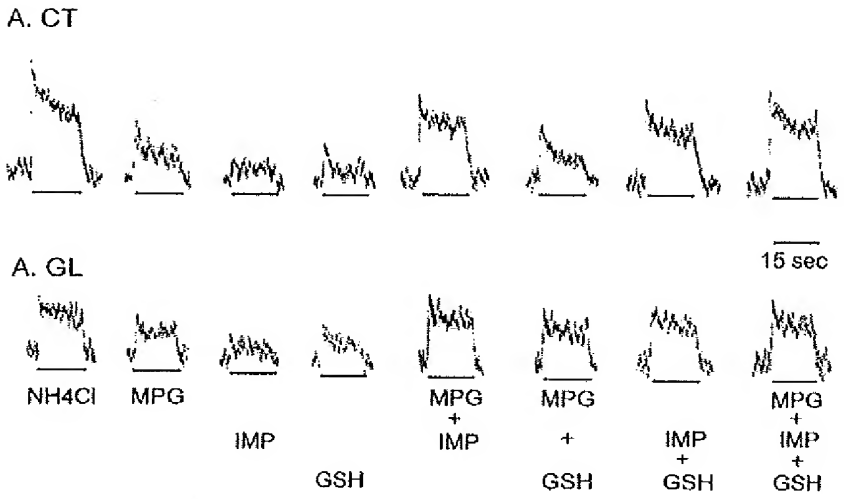

Figure 7 Representative integrated responses of the CT and the GL nerves to 0.1 M NH_4Cl, 0.1 M MPG, 0.01 M IMP, 0.01 M GSH, and 4 kinds of mixtures.

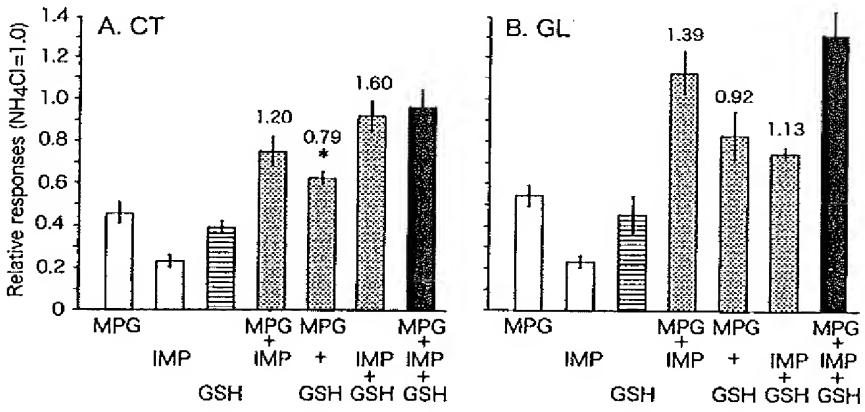

Figure 8 Mean relative responses ± SE to GSH, umami substances, and their mixtures in the CT and GL nerves. Values above each bar show potentiation ratios. The mixtures, MPG + IMP and IMP + GSH, showed large potentiation ratios, that is, the mixture responses were significantly larger than the arithmetic sum of the responses to the individual components. $*P < 0.05$, $**P < 0.01$, *t*-test.

MPG + IMP + GSH were 1.37 ± 0.17, 0.91 ± 0.15, 1.13 ± 0.13, and 1.30 ± 0.16, respectively. Potentiation ratios for MPG + IMP, MPG + GSH, IMP + GSH, and MPG + IMP + GSH were 1.71 ± 0.20, 1.02 ± 0.27, 1.66 ± 0.10, and 1.12 ± 0.21, respectively. Responses to both MPG + IMP and IMP + GMP were significantly higher than those to the arithmetic sum of the individual components ($P < 0.05$, *t*-test), indicating the occurrence of synergism. There was no significant difference between the response to MPG + GSH and the arithmetic sum of the MPG and GSH responses ($P > 0.05$).

In both nerves, the potentiation ratios for the trinary mixture exceeded 1.0, indicating a tendency of synergism. However, the values were not statistically significant in comparison to the arithmetic sum of the 3 component responses, possibly because of the ceiling effect.

Discussion

Ueda et al. (1997) found in a human sensory test that GSH induced a characteristic taste reinforcement in terms of thickness, continuity, and mouthfulness when it was added to umami solutions or a model beef extract. In other words, under the action of GSH, hedonically positive aspect of umami taste is enhanced, continues, and spreads within the whole mouth. Such characteristics are collectively called kokumi in Japanese. Kokumi may be a result of processing of afferent information within the higher center of the gustatory system

as well as gustatory interaction at the peripheral receptor level. The results of Ueda et al. also suggest that one of the essential kokumi actions is based on the umami substances. The present study is the first attempt to reveal the nature of kokumi on the basis of behavioral and electrophysiological studies in animals.

C57BL/6 mice showed no particular preference for aqueous solutions of GSH compared with dw in the 2-bottle preference test at low (0.1 and 0.3 mM) and high (30 mM) concentrations, but they preferred the solutions at concentrations ranging from 1 to 10 mM. In a human sensory evaluation test, however, GSH elicited no remarkable taste except sourness in water because of its acidic nature (Ueda et al. 1997). Ueda et al. (1997) reported that the threshold for GSH in inducing the kokumi effect was 0.04% (about 0.8 mM), corresponding well with the preference threshold in mice, which occurs between 0.3 and 1 mM.

MSG evokes both sodium and umami tastes in rodents and dogs (Sato and Akaike 1965; Ninomiya and Funakoshi 1987; Kumazawa and Kurihara 1990; Yamamoto et al.. 1991; Grobe and Spector 2008). In the present study, therefore, we used MPG instead of MSG to avoid possible influences of sodium taste on the taste of glutamate. The taste solution used in the present behavioral study was mostly fixed to one concentration for each stimulus. Although this is a limitation for a complete experiment with a wide range of concentrations, we selected 0.1 M MPG and 1 mM IMP, because these stimuli were commonly used for the umami study in rodents and induced dominant synergy when a mixture containing these solutions was used (Ninomiya and Funakoshi 1987; Yamamoto et al. 1991; Sako and Yamamoto 1999), and 1mM GSH, because this concentration was near the threshold for taste effectiveness as described above for both mice and humans.

The present short-term (10 min) and long-term (48 h) 2-bottle tests showed that the umami solutions, IMP and a mixture of IMP and MPG, were preferred more in the presence of GSH. These results may not be explained simply by the addition of the preferable component of GSH to the taste of umami solutions because the addition of GSH to MPG did not increase the preference for this mixture. It is difficult to determine which component, that is, GSH, IMP, or MPG, is influenced and shows increased taste intensity when mixed. Taste quality may change depending on the combination of the 3 chemicals because the taste of IMP may not be identical to the taste of glutamate as suggested in rats (Witfall et al. 2007).

To investigate whether the enhanced preference caused by the addition of GSH was elicited by the taste effect or a postingestive effect, we conducted the short-term 2-bottle preference test in naive mice and the long-term preference test in mice with the taste nerves transected. The results of these 2 experiments suggested that the GSH effects were mainly due to the taste effect. However, we cannot exclude possible influences of postingestive factors because of the lack of a well-defined preference for IMP + GSH over IMP, whereas statistically significant, in the short-term test, and the tendency of persistence of preference even after the CT and GL were transected. Alternatively, the persistence of preference after denervation of the CT and GL may be attributed to umami responses of the greater superficial petrosal nerve innervating the palatal taste buds (S. Harada, personal communication).

The lack of an additive effect of GSH on the preference for MPG, but not for IMP, which was confirmed at different concentrations of MPG and IMP, suggests that GSH exerts its taste effect by binding to common receptor sites with MPG, but not with IMP, indicating a kind of competitive interaction between GSH and MPG. GSH is a tripeptide with glutamic acid in its chemical structure, so this part may interact with the same receptor sites as those for glutamate of MPG. The lack of synergism in the trinary mixture of GSH, MPG, and IMP might be due to the ceiling effect. The taste effectiveness of GSH under the presence of IMP might be explained by binding of GSH to possible exposed receptor sites for glutamate as a result of the action of IMP, the idea being proposed by Torii and Cagan (1980). Alternatively, as a recent molecular study (Zhang et al. 2008) suggests, IMP may trap both glutamate and/or GSH to synergistically increase their responses.

To investigate whether GSH elicits a synergistic effect at the taste receptor level, we recorded taste nerve responses of the CT and GL to umami substances, GSH, and their mixtures. Both nerves showed essentially the same response characteristics to these taste stimuli, and the noticeable findings were that the potentiation ratios for MPG + IMP and IMP + GSH, but not for MPG + GSH, exceed 1.0, suggesting that GSH elicits synergism with IMP but not with MPG. The lack of synergism between GSH and MPG is comparable to the lack of an additive effect of GSH on the preference for MPG in the present behavioral experiments. Although the additive effects of GSH on umami responses were not identical in these 2 taste nerves, the difference may not be significant because mice without either of these nerves showed essentially the same result (see Figure 5). Both nerves are important in exerting the GSH effect because the simultaneous denervation of the 2 nerves was effective in abolishing the effect.

If GSH and MPG have common binding sites in taste receptors, the taste of GSH may be similar to that of MPG. This assumption was partly proved in the present CTA experiment in which mice trained to reject MPG also rejected GSH although the degree of rejection was not so strong. However, the reverse was not the same, that is, mice trained to reject GSH did not reject any other taste stimuli including MPG and IMP as compared with the control group, indicating that the taste of GSH is unique and is independent of other tastes. Although we have to confirm these results with different concentrations of each tastant in future experiments, the results suggest that GSH also has interactions

with other taste receptors than those for the 5 taste receptors including glutamate receptors. One possibility is an interaction with the calcium-sensing receptor (CaSR) because Wang et al. (2006) identified that GSH acted as a potential ligand to the rat CaSR and showed that GSH acted as a potent enhancer of calcium-induced activation of the CaSR. Recently, San Gabriel et al. (2009) actually found CaSR in a subset of cells in circumvallate and foliate papillae, with fewer cells in the fungiform papillae, isolated from rat and mice.

It is not known whether rodents are good models for the study of kokumi because of the species difference of the umami receptor, T1R1 and T1R3, that is, this receptor responds to various amino acids including glutamate in rodents (Nelson et al. 2002), whereas it functions as a much more specific receptor, responding selectively to MSG and aspartate in humans (Li et al. 2002). We conducted the present experiments in C57BL mice to obtain any hints about the nature of kokumi, that is, enhanced taste reinforcement accompanied by thickness, continuity, and mouthfulness in a simplified experimental setup consisting of GSH as a kokumi-inducing substance and umami substances such as MPG and IMP as taste stimuli. Our results showed that GSH had a preferable taste for mice in comparison to humans who were insensitive to GSH (Ueda et al. 1997). The results also showed that the mixture of GSH with IMP, but not with MPG, was more preferred than was each component of the mixture and showed a synergistic taste nerve response to a mixture of GSH and IMP. These findings, along with the fact that umami is very important in increasing the deliciousness of food (Yamaguchi 1987, 1998), suggest that GSH enhances deliciousness induced by umami substances. A relevant conclusion is that the enhanced umami response plays at least in part an important role in the kokumi-inducing action of GSH.

Further studies are necessary to elucidate the underlying receptor mechanisms of taste characteristics with the use of other kokumi-inducing substances in different species of animals and also to elucidate the mode of interaction and integration of a range of sensory information produced in the brain regions responsible for rewarding and emotional processing during ingestive behavior.

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EXHIBIT A

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